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14. ABSTRACT

We have shown a clear time dependence on the gene expression in the skin of the Tsk2/+ mice. The mouse most resembles human SSc at a narrowly defined time point (4 wks of age) which means studies that use this model as a surrogate for human SSc, must use specific time points in their analysis. We have pinpointed at least one candidate gene in the interval for Tsk2/+ and have confirmed the sequence difference between Tsk2/+ and the parent strain, 101/H. We present preliminary results on the expression of TGFβ mRNA from cells cultured on ECM from Tsk2/+ and WT littermates that suggest a mechanism for the up-regulation of TGFβ seen in the mutant strain. We show that elastin content in the skin, known to be controlled by TGFβ and possibly up-regulated in SSc is the earliest indicator of tight-skin in the tissue

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INTRODUCTION

Tsk2 mice were discovered more than two decades ago when progeny of a 101/H male in an ENU mutagenesis experiment were noted with very tight skin. *Tsk2* is homozygous lethal, similar to the *Tsk1* mouse model of SSc which results from duplication of the fibrillin gene. Tsk1 has been one of the most commonly used models for SSc and therefore has been extensively characterized. The SSc-like traits in Tsk2/+ heterozygotes are highly penetrant. In addition to a readily apparent skin fibrosis resulting from ECM anomalies, Tsk2/+ mice show more autoimmune and inflammatory features than Tsk1/+, and their longer lifespan and immune features that closely resemble human SSc features are ideal for use as a pre-clinical model. The Tsk2 mutation has been bred onto a homogeneous inbred (C57Bl/6, or B6) background in Dr. Blankenhorn's laboratory (our partnering PI on this project). B6.Tsk2/+ mice have many features of the human disease, including tight skin, dysregulated extracellular matrix deposition, and significant autoimmunity. We have found that Tsk2-mediated autoimmune and fibrotic signs develop *progressively* with age and manifest differently in females than males, a phenomenon also observed in human SSc. These SSc phenotypes in B6.Tsk2 mice are all likely due to a single genetic mutation, which remains unidentified. We proposed to identify the *Tsk2* gene and understand its mechanism of action as outlined in our statement of work (SOW). This mouse affords a unique opportunity to examine the pathways leading to the multiple clinical parameters of fibrotic disease.

BODY

Milestones were assigned to this proposal, with tasks to be accomplished by each investigator. The **summary** of our progress relative to these tasks is given below, followed by a complete discussion of our work this year.

Milestone 1 Identify Tsk2/+ gene:

Task 1 was for the Blankenhorn laboratory to collect DNA for sequencing (Months 1-6), which we have done. We were unable to collect homozygous DNA from Tsk2/Tsk2 homozygous embryos, however, as all the embryos we collected at the eight day stage, the earliest we could do with the help of a microscope, turned out to be either heterozygous Tsk2/+ or +/+ (wild-type, or WT). This collection was attempted from five Tsk2/+ females pregnant by Tsk2/+ males, so that is Tsk2/Tsk2 embryos were viable, we should have collected 25%. We believe that the age of death on the B6 background must therefore be prior to day 8 post-conception. Nevertheless, we proceeded to collect the Tsk2/+ and 101/H (parental strain) DNAs for sequencing, as the 454 sequence analysis can accurately report sequences from both chromosomes in heterozygous samples.

Task 2 (Months 6-12) was to select anchor sequences for Nimblegen chip design, so that chromosome 1 DNA in the Tsk2/+ interval could be sequenced. This was done by our subcontractor at ASRI, Dr. Fen Hu.

Task 3 (Month 6-12): Dr. Hu and her colleagues have hybridized the mouse genomic DNA to the chips and collected Tsk2/+ interval DNA, meeting this target. They have sequenced both Tsk2/+ and 101/H interval DNA.

Task 4: Dr. Hu and her colleagues are assembling sequence data now, and will align the sequences to compare and report all observed polymorphisms. This task is in progress. This task was originally scheduled for year 1, but it will take some portion of year 2 to present the final alignment.

Milestone 2 Determination of mechanism of action of Tsk2/+ gene:

Task 1 (Months 18-32): Drexel was to breed Tsk2/+ mice to a knockout mouse with a deficiency in the newly-identified *Tsk2* gene, to determine if either Tsk2 or wild-type allele can complement the genetic deficiency. The Blankenhorn laboratory has purchased and bred three Col3A1 KO male mice to Tsk2/+ dams in July 2012 (month 12). We are expecting pups in 2-3 weeks, so results are not yet available.

Task 2 (Months 1-36). Correlate the known actions of the Tsk2 gene at Drexel with gene expression data at Dartmouth (Aim 2) and with the presence of proliferating cells (Aim 3). In this Task, largely accomplished at Dartmouth with the microarray studies (months 4-12), we will establish the timeline for

the gene signatures in male and female Tsk2/+ mice. We will then examine the corresponding Tsk2/KO mice for these phenotypes to detect alterations in the TGFβ 1-driven proliferative, fibrotic signature of the Tsk2/+ gene when it is absent. We expect that TGFβ1 is a necessary component in the disease pathway, so at Drexel, we will breed Tsk2/+ mice to TGFβR conditional KO mice when we fully understand the timeline of the TGFβ signature.

Milestone 3 Determine the timing of TGFβ activation in the Tsk2/+ mice, and differences between males and females. Dr. Blankenhorn will send mouse tissues to Dr. Whitfield, who will do the RNA work.

Task 1 (Months 1-36): The Blankenhorn laboratory will breed sufficient numbers of mice to collect skin at postnatal Day 0, day 7, day 14, day 21 as well as 1 month and 4 months. These mice are used by all three investigators, and whenever possible, each individual mouse was studied for the relevant traits in each laboratory, so that histology and RNA transcript analysis will occur on the same animal. We have met our targets in year 1.

Task 2 (Months 4-12) Prepare RNA from skin at Drexel and hybridize DNA microarrays at Dartmouth. Data will be analyzed, processed and stored. In practice, we found it better to send whole skin samples to Dartmouth and prepare the RNA there.

Task 3 (Months 12-36): At Dartmouth perform data analysis for expression of TGFβ as well as other gene signatures, both profibrotic (IL13 and IL4) and those that may not be expected (genome-wide).

Task 4 (12-24 months, if necessary): If the microarray study is unclear, we had proposed a small number of RNASeq runs to validate the gene expression data.

Task 5 (dependent timing): Immunohistochemistry will be performed for the validation of TGFβ signatures found in the microarrays. This will be performed by the Artlett and Blankenhorn laboratories. This study relies on the completion of Aim 2 (Milestones 2 and 3), for which we need microarray data from all ages.

Milestone 4 Characterize how well the Tsk2/+ mouse approximates human SSc at different time points.

Task 1: At Dartmouth, map mouse genes to human orthologs, integrate mouse and human data using Distance Weighted Discrimination to remove systematic biases, and cluster mouse and human data (months 12-36).

Task 2: At Dartmouth, Analyze data-driven groupings, pathways, computational validation and data interpretation (months 12-36). Data analysis for expression of proliferative signatures will give us a way to understand the subset of SSc patients that exhibit diffuse clinical symptoms with signs of cell proliferation. This is a special investigation of proliferative signatures by the Whitfield group to capitalize on their extensive experience with cell cycle and proliferative motifs in gene expression. It was scheduled for months 8-24, and is in process.

Milestone 5 Experimental (functional) validation.

Task 1: We will perform confirmation qRT-PCR on select genes based on Aim 2 in the Blankenhorn and Whitfield laboratories. We had scheduled this for months 4-24; and thus, we have not completed this task as microarray data from all ages is not yet finished. In retrospect, we will modify this task to extend to year three as well, to ensure full study of interesting gene expression patterns over mouse developmental ages. In the Drexel laboratories, we plan experimentation on the mechanotension of the ECM when it contains Tsk2/+ collagen in comparison to ECM containing WT collagen, after the identification of the Tsk2/+ candidate gene by Aim 1.

Milestone 6 Cross-breed Tsk2/+ mice to Wsh mast cell knockout mice (at Drexel).

Task 1 (no date): B6.Tsk2/+ mice will be bred to B6.Wsh (c-kit deficient) mice (Jackson stock # 005051) to further determine the role of mast cells in the TGFβ1 signature observed in the Tsk2/+ mouse. We have

reason to think that this will not be necessary, as we have found no difference in the mast cell number between Tsk2/+ mice and their age and sex-matched littermates.

Preliminary results and research accomplishments

1. *Sequence of Tsk2/+ region*

We have sequenced the relevant 2 MB interval containing the Tsk2/+ gene (Drexel and ASRI). Tsk2 locus-specific capture from mouse chromosome 1 (43.9-45.9 Mb) from both tight skin-affected an unaffected animals was done using whole genome DNA and a custom Nimblegen capture array designed to provide coverage for all nonredundant sequences within the SSc locus. This array was composed of overlapping long (~70 nucleotides) oligomers that collectively cover the genomic locus (minus redundancies). This custom array provided coverage of the region between nucleotide bases 44,241,285 and 47,116,891 of the mm10_dna build of mouse chromosome 1. Thus, the total locus size is 2,875,605 bp. The capture array has probes that directly cover 56.3% of this region or 1,618,504 bp – representing the nonredundant aspects of the locus. However, the amount of DNA sequence (i.e. total length of the actually captured DNA) from each animals genomic DNA was significantly higher as the DNA fragments being captured are much longer than the probes. This resulted in the production of > 2 Mb of targeted unique sequence for each animal which was generated using the CGS's 454 LifeSciences Titanium platform. The DNA yield and the fold enrichment (based on qPCR) for the four samples prior to sequencing are as follows:

Mouse 2044 = 15.5 ug and 50 fold enrichment

Mouse 2045 = 6.4 ug and 14 fold enrichment

101B = 22.5 ug and 29 fold enrichment

101E = 23.0 ug and 34 fold enrichment

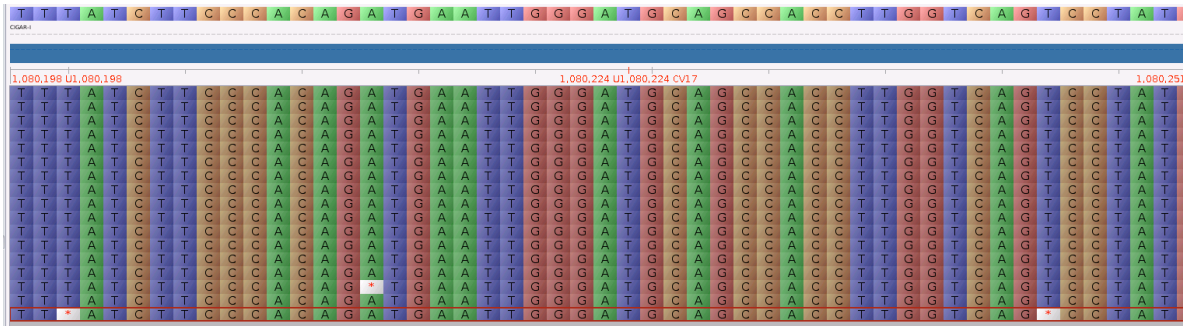
Methods: The Nimblegen sequence capture protocol began with 3-5 ug of high molecular weight genomic DNA from each animal. This DNA was used in each case to prepare a 454 LifeSciences Titanium shotgun sequencing library. From each of these libraries 15 ng of DNA was used in a ligation mediated (LM) PCR to bring the total amount of library DNA up to at ~ 3 ug. For hybridization with the SSc custom sequence capture arrays we combined: 1) 2 ug of the LM-PCR amplified library; 2) 100 ug of murine COT DNA (to hybridize with highly repetitive genomic sequences); 3) 650 picomoles of hybridization enhancing oligos; 4) 7.5 ul 2x sequence capture (SC) hybridization buffer; 5) 3 ul SC hybridization component A; and 6) 4.5 ul of each of the individual murine genomic libraries. This mixture was incubated at 47 °C for 72 hours. It was then added to 100 ul of Streptavidin Dynabeads to bind the biotin tags on the genome libraries. Incubation was at 47 °C for 45 min. The tubes were then placed in a Dynamag-2 device to bind the beads which were washed repeatedly to remove any genomic DNA that was not bound to the custom locus library array. The captured DNA was eluted and resuspended in water and then reamplified once more using LM PCR. A qPCR SYBR Green assay was used to determine the amount of enrichment by comparing samples before and after capture. This captured DNA was then subjected to 454 Titanium sequencing.

Initial assembly of the captured DNA into contigs was performed using the Newbler de novo assembler and then completeness of coverage and coverage depth were determined (see Table 1 for sequencing and alignment metrics). The contigs were then scaffolded against the latest curated mouse genome to ensure that all nonredundant sequences were captured and sequenced to a sufficient depth to ensure reliability of the data. For the four libraries we obtained between 116,000 and 157,000 reads of which 75-85% mapped to the locus indicating that the capture beads had provided excellent targeted enrichment. Our average coverage levels ranged from 4.5 – 9.6X. Although as indicated above the number of probe bases was only 1.6M, we did recover an average of > 2.1 M bases due to the large fragment sizes of the captured DNA. Our average contig size was > 2 Kb. We used Mauve to identify allelic differences between the affected and unaffected DNAs. The alignment is in progress, but based on a number of early results from excellent candidate genes, we have narrowed in on several of these. As can be seen in Table 1 and **Figure** 1, the coding exon SNP in *Col3A1* is seen

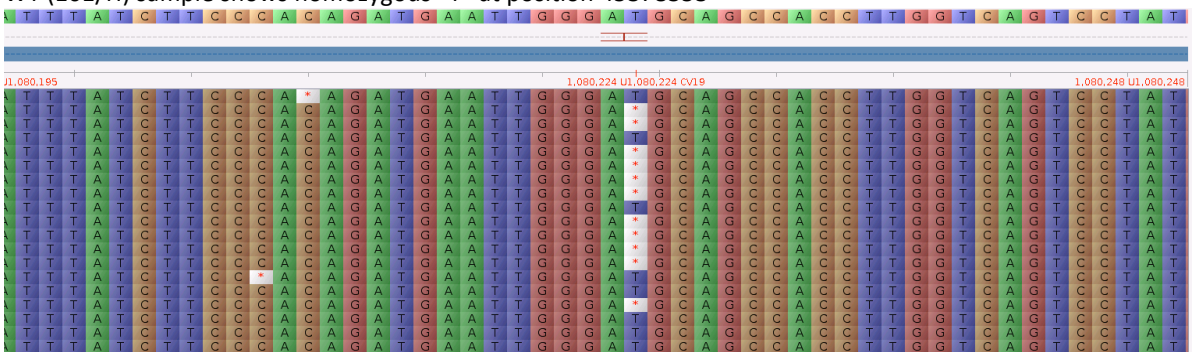
as heterozygous in Tsk2/+ samples, as expected.

Table 1. SNPs found in candidate genes.

Position	Corrected Pos	#CHROM	GWEIGHT	Gene	REF 2211 tsk2	ALT 2211 tsk2	QUAL tsk2	REF 2214 tsk2	ALT 2214 tsk2	QUAL 2214 tsk2	REF 2216 tsk2	ALT 2216 tsk2	QUAL 2216 tsk2
45880257	1582128	chr1	2	WDR75 Exon	AC	CG	2.9693	A	C	0.597642	AC	CG	19.8066
45432389	1134260	chr1	2	COL5A2 3'UTR	G	C	183.141	G	C	158.412	G	C	319.07
45378353	1080224	chr1	2	COL3A1 Coding Exon	T	A	1226.23	T	A	3032.56	T	A	289.282
45875728	1577599	chr1	2	WDR75 Coding Exon	T	C	483.524	T	C	129.314	T	C	94.8358



WT (101/H) sample shows homozygous “T” at position 45378353



Tsk2/+ sample shows heterozygosity (T + A (indicated by *)) at this position.

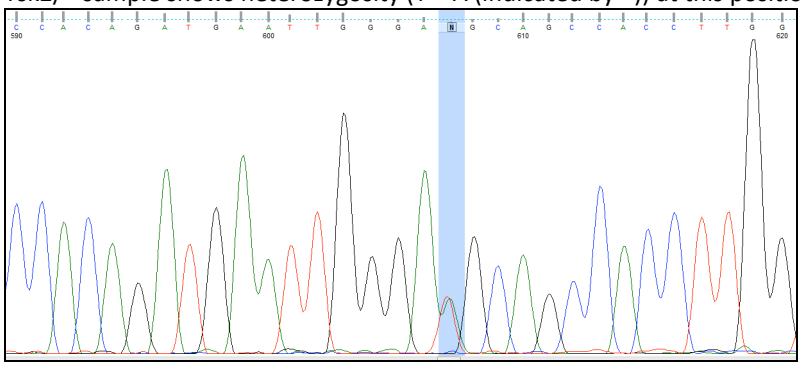


Figure 1. Individual sequence reads from 101/H (WT) sample (top) and Tsk2/+ sample (bottom). WT (101/H) sample shows homozygous “T” at position 45378353 Tsk2/+ sample shows heterozygosity (T + A (indicated by *)) at this position. Bottom panel shows confirmation in PCR amplified genomic DNA from Tsk2/+ at Drexel of the SNP found by whole interval sequencing.

We confirmed the global sequencing result at Drexel (**Figure** 1 bottom) and also evaluated the remaining SNPS by phototyping[1] . Of these, only the Col3A1 non-synonymous coding SNP was validated; two

The genome-capture sequencing result was confirmed at Drexel (Figure 1 bottom) and an the remaining SNPs evaluated by phototyping[1]. Of these, only the Col3A1 non-synonymous coding SNP validated; two intronic SNPs in the GULP1 gene also distinguish Tsk2/+ from all other strains for which chr 1 genotyping is available. The Col3a1 SNP results in a Cys to Ser change in the PIIINP (N-terminal) cleavage product of the Col3a1, and thus is a target for our research in the immediate future.

Expression of elastin

Drs. Artlett and Blankenhorn have nearly completed the study of *elastin expression* and its role in fibrosis. Elastin is a key component of the tight skin phenotype of Tsk2/+ mice. We had previously observed that abnormal collagen accumulation occurs with age in Tsk2/+ mice, but it does not occur until 10 weeks of age, fully 8 weeks after the development of the "tight skin" phenotype. While we observed transcripts of transforming growth factor (TGF)-β1 responsive genes with increased levels in Tsk2/+ skin, correlating with higher levels of TGF-β1 at 2 and 10 weeks of age, this didn't directly explain the very tight skin at two-three weeks of age.

Figure 2. Top – A, Bottom – B

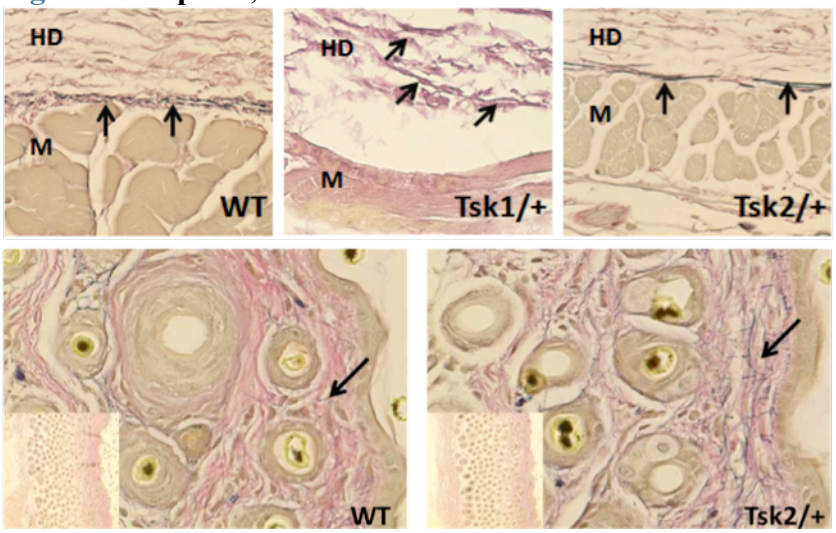


Fig 2C

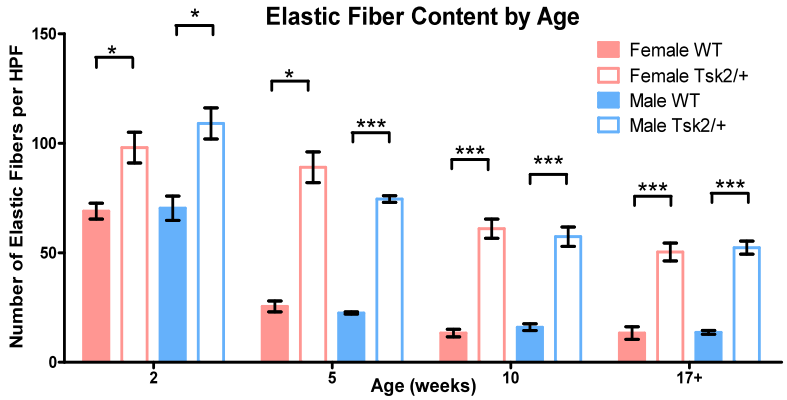
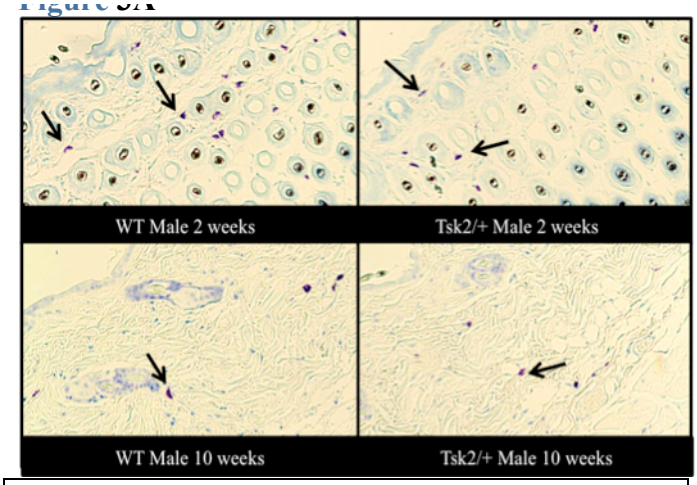


Figure 2. Tsk2/+ mice have increased elastic fibers in skin. Skin samples were stained with Weigert's Resorcin Fuchsin Stain and elastic fiber number per high powered field was calculated. **A,** Elastic fiber WT (left), Tsk1/+ (middle) and Tsk2/+ (right) male mice at 5 weeks of age (400X magnification) shown between the hypodermal muscle (M) and the hypodermal connective tissue (HD). Distinct elastin fibers are marked with arrows. **B,** Elastic fibers in the dermis from WT (left) and Tsk2/+ (right) female mice at 2 weeks of age. Images are shown at 400X magnification. **C,** Quantitative amount of elastic fibers per HPF over time. N=4-6 mice per group, 5-9 HPFs per slide. * p<0.05, **p<0.01, ***p<0.001.

We then found a highly significant increase in elastic fibers in 2-week-old Tsk2/+ mice that continues throughout adulthood. The timeline of disease development in the Tsk2/+ mouse shows that fibrosis is progressive, with novel elastic fiber changes occurring months before collagen accumulation. Tsk2/+ mice have increased dermal levels of TGF-β1 prior to the development of disease, suggesting that fibrosis is TGF-β1 driven and involves elastin, not collagen, in early stages.

Skin samples from Tsk2/+ and WT mice at 2, 4, 10, and 23 weeks of age were examined for other ECM anomalies, based on the increased expression of certain ECM genes early in life). As early as 2 weeks of age, Tsk2/+ skin had significantly more elastic fibers in the dermis compared to WT mice (Figure 2B, p<0.05). The increase in elastic fibers is maintained through all ages (Figure 2C). There was no difference in the elastic fiber layer beneath the panniculus carnosus in Tsk2/+ mice, unlike Tsk1/+ mice (Figure 2A), indicating another significant difference between the two models of disease. The difference between Tsk2/+ and WT littermates in elastic fiber expression was one of the earliest and most reliable signatures of the Tsk2/+ phenotype and, unlike collagen deposition, predicted fibrosis and the tight skin phenotype seen in Tsk2/+ skin as early as the skin pinch and foot caliper assays.



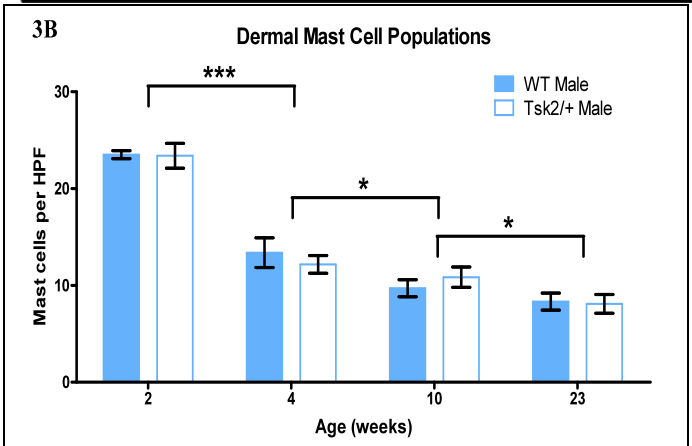


Figure 3. Infiltrating mast cell populations in Tsk2/+ male mice are similar to WT male mice. Skin sections were examined for mast cell numbers per high powered field (HPF). **A**, There is no difference in mast cell populations between Tsk2/+ and WT male mice at any age. **B**, There is a significant decrease in mast cell numbers with age. N=4-6 mice per group and 9 HPFs per slide. * p<0.05, ** p<0.01, *** p<0.001.

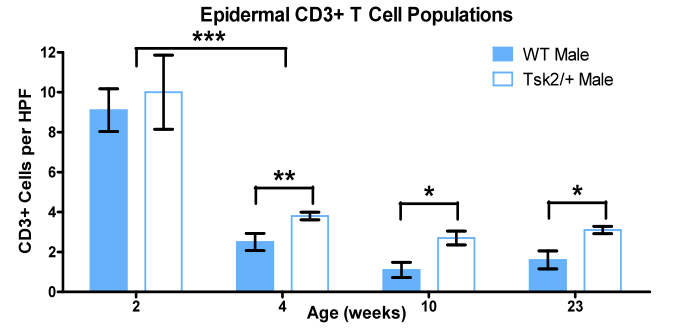


Figure 4. Epidermal CD3+ T cell populations in Tsk2/+ male mice are slightly higher than in WT male mice. Skin sections were examined for the number of CD3+ T cells per high powered field (HPF). There is a significant decrease in CD3+ T cell numbers between age 2 and 4 weeks of age. N=4-6 mice per group and 9 HPFs per slide. * p<0.05, ** p<0.01, *** p<0.001.

2. *Cellular infiltrates in Tsk2/+ mice*

In addition, we also found no substantial difference in the number of infiltrating cells in the skin of Tsk2/+ mice. Dermal mast cells were seen in equivalent numbers in Tsk2/+ and WT mice across all ages (**Figure** 3A) although all mice showed a significant decrease in mast cell numbers with age (~2-fold decrease between 2 and 4 weeks of age, p<0.0001). CD3+ T cell analysis showed a significant, yet small increase in the number of cells in the Tsk2/+ epidermis compared to WT littermates starting at 4 weeks of age (**Figure** 4). Importantly, there was no difference in CD3+ T cell number at 2 weeks of age. Like the mast cell decrease early in life, there was ~3-fold decrease in epidermal CD3+ T cells between 2 and 4 weeks of age (p<0.001).

We conclude that while there was no difference in dermal mast cell numbers, there is a very subtle increase in the number of infiltrating T cells in the skin of Tsk2/+ mice. The possible physiological impact of this infiltrate is questionable as it occurs after the tight skin phenotype is detectable. We have combined these results with material presented in the DOD grant proposal into a manuscript for the *Journal of Investigative Dermatology.*

3. *RNA gene profiling*

We have initiated the RNA gene profiling by microarray. Dr. Whitfield's laboratory at Dartmouth has focused on completion of Milestone 3, task 2 (months 4 – 12). To accomplish this milestone we analyzed skin from both wt and Tsk2/+ mice at 4, 8, 12, and 20 weeks of age. We have also paid careful attention to the differences between males and females. We have analyzed the data from our DNA microarray hybridizations and found a clear time dependence on the gene expression in Tsk2/+. Analysis of the female mice at 4 weeks of age identified a specific gene expression signature of 405 genes (FDR < 10%) as determined by Significance Analysis of Microarrays (SAM; **Figure 5A**)[2]. We find that the majority of genes upregulated in Tsk2/+ mouse skin at 4 weeks of age map to the GO Biological processes of Cell adhesion and Cell morphogenesis (DAVID, Benjamini-corrected p < 0.05). Genes that shown increased expression include Col6a1, Col6a2, Col5a1, Sparc and Thy1. Many of these genes are known targets of the profibrotic cytokine TGFbeta. Therefore, we have an initial indication that we will find high expression of TGFbeta responsive genes in mouse skin at 4 weeks of age. Therefore, we have begun to accomplish Task 3 under Milestone 3, to identify TGFbeta responsive gne signatures in the Tsk2 mouse (to be completed months 8 – 24). Genes with decreased expression at 4 wks were not enriched for any specific GO biological process or KEGG pathway.

Analysis of differential gene expression between wt and Tsk2/+ mice at 8 weeks of age did not show any statistically significant differences. In contrast, analysis of differential gene expression between wt and Tsk2/+ mice at 12 weeks of age revealed striking differences in gene expression (**Figure 5B**). We find 1100 genes differentially expression (SAM, FDR<0.2%). Analysis of the GO biological processes show the dominant program among the upregulated genes is *M phase*, *cell cycle*, *mitosis*, *DNA packing* and *chromatin assembly*, suggesting the presence of proliferating cells [3, 4]. Genes included among these processes are the cell cycle regulators CCNB1, CCNA2, CDCA2, FOXM1 and PLK1. The genes with decreased expression at 12 weeks are enriched for the GO biological process of *Immune response* (DAVID, Benjamini-corrected p<0.05) and include genes IL18 receptor 1 (IL18r1), IL1beta, ccl7 and mst1. We currently do not have enough samples at 20 wks to perform this analysis. Additional samples will be added in the coming year to bring ensure statistical power to analyze the remaining time points.

These data are consistent with our earlier results (preliminary data for this grant) that showed Tsk2/+ resembles human SSc TGFbeta activated subset at 4 weeks, but not a 16 weeks.

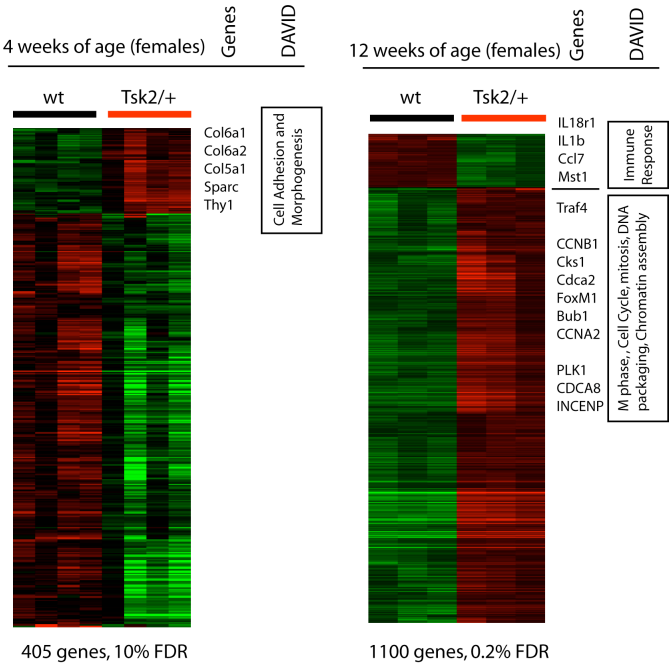


Figure 5. Differential gene expression in skin between wt and Tsk2/+ female mice during development. A. Mice at 4 weeks of age were analyzed for statistically significant differences in gene expression using significance analysis of microarrays (SAM) identifies 405 genes (FDR<10%). Gene expression is shown as a heat map with red indicating increased expression and green indicating decreased expression. Wt and Tsk2/+ are indicated. Select genes are indicated to the right of the heat map. GO biological process annotations enriched among these differentially expression genes as determined by DAVID are also indicated (Benjamini corrected p<0.05). **B.** Genes differentially expressed at 12 weeks of age as determined by SAM identifies 1100 genes (FDR<0.2%). Select genes and enriched GO Biological Processes are indicated.

4. Functional analysis of Tsk2/+ candidate gene

We have begun the analysis of Tsk2 candidate gene molecules at the protein level. We are still determining the best experimental approach to determine the potential difference in mechanotension of ECM containing collagen from Tsk2/+ vs. that containing collagen from WT. We have tried culturing the fibroblasts in flasks for 4 weeks to lay down matrix, however when we lysed the cells or trypsinized them to remove the cells, we also removed the collagen matrix. As we have previously studied the response of fibroblasts to collagen-coated dishes, we are approaching this problem by culturing Tsk2 or WT fibroblasts for 2 weeks to establish conditioned media that contains the collagen. Then 10 ml of that media containing secreted collagens was used to coat a new flask overnight. The media was removed and Tsk2/+ fibroblasts were seeded into flasks that had WT collagen, or WT fibroblasts were seeded into flasks that had Tsk2/+ collagen. Intriguingly, by this method we found that when Tsk2/+ fibroblasts were cultured on WT collagen, TGF-β1 transcripts were reduced; however, when either of the two WT fibroblast lines were cultured on collagen obtained from two different Tsk2/+ mice, TGF-β1 transcripts were increased approximately 2-fold (**Figure** 6). We will be repeating this experiment with age- and sex-defined cell lines, but so far, WT cells make more TGFβ when cultured on Tsk2/+ matrices. This is exactly what would be expected if the collagens produced by Tsk2/+ were altered by the mutation in such a way as to affect interactions with normal non-mutant cells. This finding mimics the TGF-β1 signature that is seen in the Tsk2 mice and leads us to speculate that this signature may be derived from outside/inside signaling by the collagens.

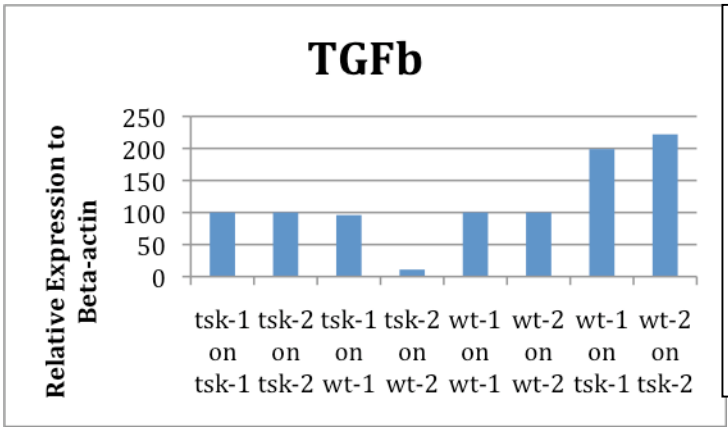


Figure 6. TGF-β1 gene expression in fibroblasts cultured on normal and Tsk2 matrix. Fibroblasts were seeded onto flasks coated with conditioned medium in a mix-and-match scheme, where cells from Tsk2/+ sample 1 were cultured on Tsk2/+ sample 1 or WT-sample 1 matrices. A duplicate experiment was performed with Tsk2/+ sample 2 cultured on Tsk2/+ sample 2 or WT-sample 2 matrices, WT-1 cells cultured on WT-1 or Tsk2/+ sample 1 matrices and WT-2 cells cultured on WT-2 and Tsk2/+ sample 2 matrices. Self combinations

Reticular Fiber Stain. We stained tissue sections from Tsk2/+ and wild-type skin for reticular fibers. Reticular fibers are comprised primarily of type III collagen and with the specific reticular fiber stain, they stain dark grey due to deposition of silver onto the fiber. In the dermis where there is less type III collagen, the collagen stains taupe/pale grey. Reticular fibers are found around the panniculus carnosus and we observed that in the Tsk2 mouse there was increased staining for reticular fibers as the mice age. We found that at 2.1 weeks of age a small staining difference was observed for Tsk2/+ in the amount of type III collagen between the muscle fibers. As the mice progressed to 10 weeks of age, the reticular fibers became less fine/fluffy and instead were thickened and the staining of the fibers was more pronounced. By 23 weeks of age, Tsk2 mice had strong staining of the collagen fibers which were substantially thickened than their wild-type littermates (Figure 7). In addition to the panniculus carnosus, we observed increased staining in the dermis for reticular fibers at age 2.1 weeks. The Tsk2/+ mice were found to have more densely packed taupe colored fibers and this was apparent from age 2.1 weeks. As the mice age through to 23 weeks, the dermis stained darker reflecting the increase in collagen III deposition. Note in the wild-type mice that the fibers are less densely packed than that observed in the Tsk2/+ mice.

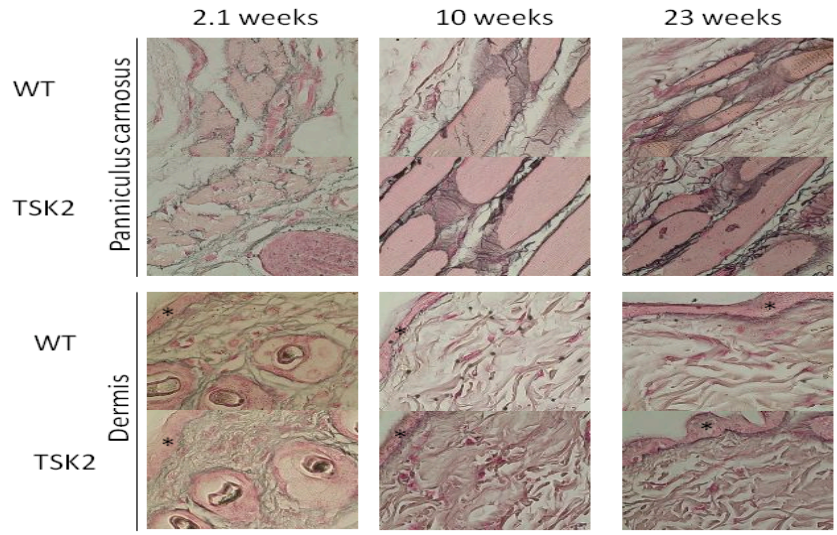


Figure 7. Reticular fiber staining in Tsk2 and wild-type mice at 2.1, 10, and 23 weeks of age. Sections were deparaffinized in 2 changes of xylene and 2 changes of ethanol, and then rehydrated. The sections were stained with 1% potassium permanganate, 3% potassium metabisulfite, ferric ammonium sulfate, and ammoniacal silver. The sections were then fixed in formalin and further stained with gold chloride and sodium thiosulfate, and then counterstained with nuclear fast red. Reticular fibers stain grey to dense black. The asterisk denotes the epidermis. All

KEY RESEARCH ACCOMPLISHMENTS

- We have started the 454 next generation sequencing of the mouse Tsk2/+ interval. Genomic DNA from two heterozygous Tsk2/+ mice and two WT littermates was sent to ASRI, and processed as described in the body of the progress report to yield four complete sequences.
- A number of single nucleotide polymorphisms have been discovered in these two new, unsequenced strains (101/H, the parental strain; and B6.tsk2, the B6 congenic strain bearing mutated 101/H DNA on chromosome 1 that contains the Tsk2/+ mutation). One of these is in *Col3A1*. This SNP and two others in intronic regions of the *Gulp1* gene have been verified.
- We have begun the breeding necessary for the genetic complementation test of *Col3a1* by breeding the Tsk2/+ line to BALB.Col3A1KO mice (heterozygotes as well). Results should be definitive and are expected soon.
- We have verified early results that the excess deposition of collagen matrix does not occur until well after the Tsk2/+ tight skin phenotype is evident. We have also verified that elastin is highly significantly increased in Tsk2/+ mice at the time the phenotype is present.
- We have new results suggesting that the collagen that is deposited in reticular fibers has a different appearance, even at early stages before frank fibrosis occurs in Tsk2/+ mice.
- Microarray profiling of Tsk2/+ mice at 4, 8, 12 and 20 weeks have been performed. We have identified genes that are differentially regulated at each time point. Tsk2/+ mice have a TGFβ1 signature that is seen in a global assessment of mRNA from skin in a carefully controlled study using littermates (Tsk2/+ and WT) at timed stages and stratified by sex.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations

Manuscripts:

Kristen B. Long, Carol M. Artlett, and Elizabeth P. Blankenhorn. Tight Skin 2 Mice have Increased TGF-β1 Levels Prior to Disease Development leading to Excessive Elastic Fibers in Skin. Manuscript to be submitted to *J. Invest. Dermatol*., 2012

Presentations:

C.M. Burgwin, K.B. Long, Zhenghui Li*, C.M. Artlett, M. Whitfield*, and E.P. Blankenhorn. "Mapping of the Mutation in the Tight Skin 2 model of systemic sclerosis" Department of Microbiology and Immunology, Drexel Univ. College of Medicine, Philadelphia, PA. *Dartmouth Medical School, Hanover, NH, Institute for Molecular Medicine and Infectious Disease International Symposium, Philadelphia, PA, June 2012

Burgwin, C.M., K.B. Long, Z. Li., C.M. Artlett, M.L. Whitfield* and E.P. Blankenhorn. "Mapping of the mutation in the Tight Skin 2 mouse model of systemic sclerosis." Department of Microbiology and Immunology, Drexel Univ. College of Medicine, Philadelphia, PA. *Dartmouth Medical School, Hanover, NH, 21st Annual Infection and Immunity Forum, Eastern PA Branch of ASM, Drexel University College of Medicine, Philadelphia, Pennsylvania, June 2012.

John, A.K., K.B. Long, L. Cort and E.P. Blankenhorn. "Fibroblast investigations suggest that increased collagen production is cell-autonomous in the Tsk2/+ mouse model of scleroderma." Drexel University College of Medicine, Discovery Day Research Symposium, Philadelphia, Pennsylvania, October 2011.

Long, K.B., C.M. Burgwin, C.M. Artlett and E.P. Blankenhorn. "Examination of pre-fibrotic and fibrotic changes in Tsk2/+ mice uncovers a novel time line of disease development and a possible new phenotype in human disease." Platform Oral Presentation, Discovery Day Research Symposium, Drexel University College of Medicine, Philadelphia, Pennsylvania, October 2011.

Long, K.B., C.M. Burgwin, C.M. Artlett and E.P. Blankenhorn. "Examination of pre-fibrotic and fibrotic disease in Tsk2/+ mice shows early ECM changes are attributable to elastin dysregulation." 12th International Workshop on Scleroderma Research, Cambridge, UK, July 2011.

Degrees obtained that are supported by this award

Kristen B. Long in Dr. Blankenhorn's lab successfully defended her PhD thesis, entitled "Examination of a model of systemic sclerosis, the Tight Skin 2 mouse: before, during and after fibrotic disease" in April, 2012. She was awarded her PhD by Drexel University College of Medicine in May 2012. Her work was directly supported by this grant to partnering PI Dr. Blankenhorn.

Development of cell lines, tissue or serum repositories

Partnering PI Dr. Blankenhorn has developed a large number of Tsk2/+ and WT littermate fibroblast cell lines from mice of various ages and both sexes.

CONCLUSION

We have shown a clear time dependence on the gene expression in the skin of the Tsk2/+ mice. The mouse most resembles human SSc at a narrowly defined time point (4 wks of age) which means studies that use this model as a surrogate for human SSc, must use specific time points in their analysis. We have pinpointed at least one candidate gene in the interval for Tsk2/+ and have confirmed the sequence difference between Tsk2/+ and the parent strain, 101/H[5]. We present preliminary results on the expression of TGFβ mRNA from cells cultured on ECM from Tsk2/+ and WT littermates that suggest a mechanism for the up-regulation of TGFβ seen in the mutant strain. We show that elastin content in the skin, known to be controlled by TGFβ[6] and possibly up-regulated in SSc[7], is the earliest indicator of tight-skin in the tissue.

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